THE FINAL (3) *REPORT ON*

CONTRACT NO DA 92 - 557 - FEC - 35675

INCLUSIVE DATES November 1 1961 *TO* October 31 1962

SUBJECT OF INVESTIGATION

EXPLORATION OF NEW CHEMOTHERAPEUTICS
FOR INFECTIOUS DISEASES

RESPONSIBLE INVESTIGATOR

Dr. Toju Hata, M.D.
President
Kitasato Institute for Infectious Diseases
Head of Antibiotic Division of
the said Institute

U.S. Army Research & Development Group (9852) (Far East)
Office of the Chief of Research and Development
United States Army
APO 343

AD Div 16/2

Kitasato Institute for Infectious Diseases, Japan
EXPLORATION OF NEW CHEMOTHERAPEUTICS FOR INFECTIOUS DISEASES by Toju Hata.
Final Report No. 3, 1 Nov 61 - 31 Oct 62. 25 p. incl. illus. tables, 5 refs.
(Contract DA 92-557-FEC-35675) Unclassified report.

Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba hystolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidones:

UNCLASSIFIED

1. Protomycine.
2. Cephalomycin.

I. Title: Chemotherapeutic Agents.
II. Hata, Toju
III. U.S. Army Research and Development Gp (FE), OCRD, DA, Wash, D. C.
IV. Contract DA 92-557-FEC-35675.

Armed Services Technical Information Agency

UNCLASSIFIED

AD Div 16/2

Kitasato Institute for Infectious Diseases, Japan
EXPLORATION OF NEW CHEMOTHERAPEUTICS FOR INFECTIOUS DISEASES by Toju Hata.
Final Report No. 3, 1 Nov 61 - 31 Oct 62. 25 p. incl. illus. tables, 5 refs.
(Contract DA 92-557-FEC-35675) Unclassified report.

Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba hystolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidones:

UNCLASSIFIED

1. Prot...ycine.
2. Cephalomycin.

I. Title: Chemotherapeutic Agents.
II. Hata, Toju
III. U.S. Army Research and Development Gp (FE), OCRD, DA, Wash, D. C.
IV. Contract DA 92-557-FEC-35675.

Armed Services Technical Information Agency

UNCLASSIFIED

AD Div 16/2

Kitasato Institute for Infectious Diseases, Japan
EXPLORATION OF NEW CHEMOTHERAPEUTICS FOR INFECTIOUS DISEASES by Toju Hata.
Final Report No. 3, 1 Nov 61 - 31 Oct 62. 25 p. incl. illus. tables, 5 refs.
(Contract DA 92-557-FEC-35675) Unclassified report.

Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba hystolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidones:

UNCLASSIFIED

1. Protomycine.
2. Cephalomycin.

I. Title: Chemotherapeutic Agents.
II. Hata, Toju
III. U.S. Army Research and Development Gp (FE), OCRD, DA, Wash, D. C.
IV. Contract DA 92-557-FEC-35675.

Armed Services Technical Information Agency

UNCLASSIFIED

AD Div 16/2

Kitasato Institute for Infectious Diseases, Japan
EXPLORATION OF NEW CHEMOTHERAPEUTICS FOR INFECTIOUS DISEASES by Toju Hata.
Final Report No. 3, 1 Nov 61 - 31 Oct 62. 25 p. incl. illus. tables, 5 refs.
(Contract DA 92-557-FEC-35675) Unclassified report.

Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba hystolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidones:

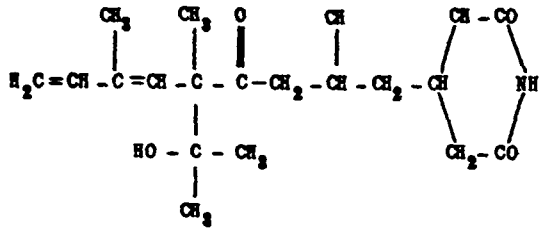
UNCLASSIFIED

1. Protomycine.
2. Cephalomycin.

I. Title: Chemotherapeutic Agents.
II. Hata, Toju
III. U.S. Army Research and Development Gp (FE), OCRD, DA, Wash, D. C.
IV. Contract DA 92-557-FEC-35675.

Armed Services Technical Information Agency

UNCLASSIFIED



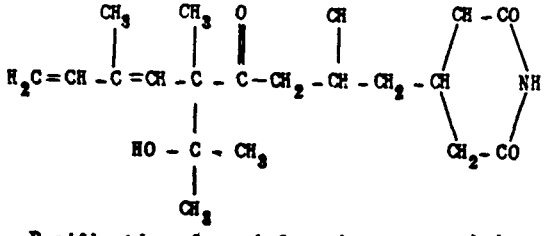
Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylalanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined. (Author)

UNCLASSIFIED

DESCRIPTORS

Chemotherapeutic Agents
Culture Media
Antibiotics
Diseases
Infections
Streptomyces
Medical Research
Streptomycins
Japan

UNCLASSIFIED



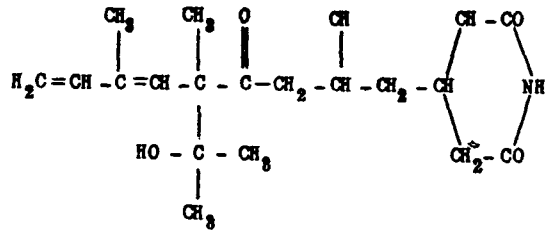
Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylalanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined. (Author)

UNCLASSIFIED

DESCRIPTORS

Chemotherapeutic Agents
Culture Media
Antibiotics
Diseases
Infections
Streptomyces
Medical Research
Streptomycins
Japan

UNCLASSIFIED



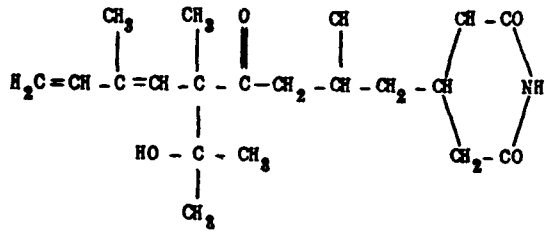
Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylalanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined. (Author)

UNCLASSIFIED

DESCRIPTORS

Chemotherapeutic Agents
Culture Media
Antibiotics
Diseases
Infections
Streptomyces
Medical Research
Streptomycins
Japan

UNCLASSIFIED



Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylalanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined. (Author)

UNCLASSIFIED

DESCRIPTORS

Chemotherapeutic Agents
Culture Media
Antibiotics
Diseases
Infections
Streptomyces
Medical Research
Streptomycins
Japan

UNCLASSIFIED

D-I-S-T-R-I-B-U-T-I-O-N

The distribution of this report as made by USA R&D Gp (FE)(9914) is as follows:

Army Research Office, OCRD, Washington 25, D. C.	(3)
Army Attache, American Embassy, Tokyo, Japan	(1)
U.S. Army Medical R & D Command	(4)
ASTIA	(10)

EXPLORATION OF NEW CHEMOTHERAPEUTICS FOR INFECTIOUS DISEASES

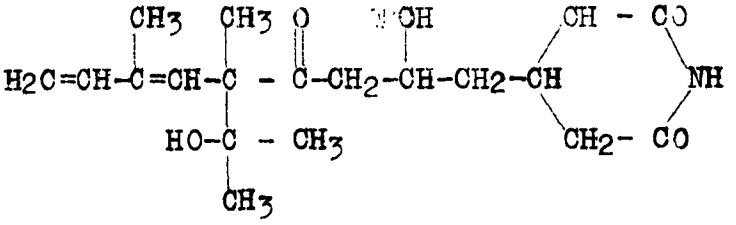
Fundamental Studies on Protomycin, an Antiamaebic Antibiotic and Cephalomycin, an Antiviral Antibiotic

Toju Hata
Ryozo Sugawara, Akihiro Matsumae
and
Hiroshi Yamamoto

Kitasato Institute
for
Infectious Diseases
Tokyo, Japan

ABSTRACT

Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba histolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidones:



Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylalanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined.

Content **Page**

1. Introduction

Fundamental researches on protomycin, a new antibiotic active against saccharomyces and Endameba histolytica, and cephalomycin, a new antibiotic against Japanese B. encephalitis virus, respectively.were carried out.

To know the structure of protomycin in reference to the established structure of streptimidone, another antibiotic resembling to protomycin found by Dr. Frohardt et al in Parke Davis, the degradation products from protomycin were compared with corresponding ones simultaneously obtained from streptimidone.

Since cephalomycin is a polypeptide neither crystallized nor assured for homogeneity, further purification was attempted to increase its unitary activity by conventional procedures for purifying protein.

2. Experimentation

a. Protomycin, degradation products

Reference to physico-chemical and biological properterties, particularly to the result of elemental analysis, infrared and antibacterial spectra of protomycin (1), suggested it to be an antibiotic of cycloheximide series.

For obtaining some knowledges on the structure of protomycin as related with streptimidone[2][3][4], another antibiotic of cycloheximide group with the established structure, several degradation products from protomycin were compared with corresponding ones from streptimidone.

Protomycin (I), a pale yellow viscous liquid, was purified by molecular distillation and proved to be homogeneous by counter-current distribution. Although recrystallization from a solution was so far unsuccessful, it changed into a white crystals, M.P. 58-61 C, when allowed to stand several weeks in refrigerator. The elemental analysis satisfied the empirical formula of $C_{16}H_{29}NO_5$. Ultraviolet spectrum possessed maxima at 232.5 mμ (ε=24,300) and 287 mμ (ε=1,440) in methanol. Specific optical rotation is $[\alpha]_D^{27}$=126° (c=1.08, $CHCl_3$). Infrared absorption maxima in nujol mull were found at 2.71, 2.81, 3.07, 3.13, (3.40), 5.78, 5.88, 6.09, 6.21, (6.90), (7.30), 7.75, 7.98, 8.12, 8.35, 8.50, 8.69, 9.12, 9.49, 9.75, 9.88, 10.10, 10.98, 11.15, 11.23, and 11.45 μ. The molecular formula is analogous to those of the antibiotics

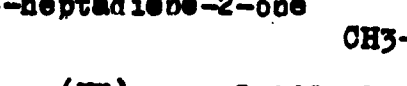
of cycloheximide series. The ultraviolet spectrum, suggesting the existence of a conjugated double bond, is closely related with that of streptimidone(2)(3)(4).

The overall form of the spectrum corresponds well to that of streptimidone, except a marked difference in the neighbourhood of 8μ and differences in details in the region lower than 3.0μ and fingerprint region.

Protomycin absorbs 2.08 moles of hydrogen when hydrogenated in glacial acetic acid or ethyl alcohol with Pd-on-carbon catalyst and gives tetrahydroprotomycin (II).

The product, while first viscous, changed into white crystals, M.P. 39-44°C, when allowed to stand in refrigerator. The elemental analysis satisfied the empirical formula $C_{19}H_{33}NO_5$. Ultraviolet absorption maximum is at 282 mμ ($E_{1cm}^{1\%}$=203) in ethanol. The peak in the infrared region were found at 2.99, 3.02, 3.15, 5.78, 5.81, 5.90, 5.95, 6.60, 6.68, 6.90, 7.30, 7.65, 7.75, 7.80, 7.92, 8.08, 8.39, 8.49, 8.68, 8.78, 9.02, 9.20, 9.32, 9.50, 9.73, 9.81, 10.29, 10.72, 10.80, 10.86, 11.35, 11.50, 11.75, 11.98, and 12.18 (in major). While the characteristic absorption bands at about 8μ unchanged, the bands at 6.09, 6.21, 10.10 and 10.98 disappeared and in conjunction with the change in ultraviolet spectrum, the presence of a conjugated diene in the original protomycin was definitely suggested.

Protomycin was steam distilled from a solution in 4% NaOH into 2N-HCl, saturated with 2,4-dinitrophenylhydrazine. The resulting suspension was extracted with benzene and fractionated through a column of silicic acid, with benzene as a developing solvent. A dark red crystal and an orange crystal were obtained; the former, M.P. 165°C and molecular formula $C_{15}H_{18}N_4O_4$, was identified with the corresponding product from streptimidone and the latter, M.P. 122°C, with the 2,4-dinitrophenylhydrazone of acetone by the mixed melting point, elemental analysis and infrared spectrum respectively. Accordingly, protomycin gives 3,5-dimethyl-3,5-heptadiene-2-one

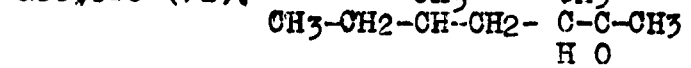


(III), and acetone (IV) as volatile ketones by treating with alkali.

By the same procedure, tetrahydroprotomycin (II) gave two kinds of yellow crystals and one orange crystals of 2,4-dinitro-phenylhydrazone.

One of the yellow crystals, M.P. 59-64°C and molecular formula $C_{15}H_{22}N_4O_4$, was identified with the

corresponding product from streptimidone; the other yellow crystals, probably a stereoisomer of the former, showed M.P. 96-98°C, while it satisfied the same molecular formula. The orange crystals were proved again to be the derivative of acetone. Thus, the volotile ketones obtained by the alkaline treatment of tetrahydroprotomycin were found to be 2,5-dimethyl-2-heptanone (V) and acetone (VI).



The ozonide of protomycin was prepared in methanol, decomposed with an aqueous ferrous sulfate solution and steam distilled into 2N-HCl saturated with 2,4-dinitrophenylhydrazine. The precipitate was purified by silicic acid chromatography and identified with the derivative of formaldehyde. The residual solution was filtered, added with an aqueous alkaline solution and steam distilled. From the precipitate of 2,4-dinitrophenylhydrazone, the derivatives of methyl ethyl keton and acetone were isolated by silicic acid chromotography and identified with authentic samples, respectively. When ozonide was prepared in chloroform and decomposed with water, there were proved again formaldehyde and acetoaldehyde from direct steam distillate and methyl ethyl ketone and acetone from steam distillate from alkaline solution

Tetrahydroprotomycin (II) was converted into oxime with hydroxylamine-pyridine in ethanol. The reaction mixture remained viscous in spite of several procedures of purification, so that it was treated with 10 ml of 75% H_2SO_4 on boiling water bath to induce Beckmann's rearrangement. The reaction mixture was diluted with 20 volumes of water and steam distilled. The oily acid in the distillate was treated with p-phenylazophenacylbromide. The resulting ester (VII), orange colored and melting at 54-55°C, satisfied an empirical formula, $C_{25}H_{30-32}N_2O_4$. Thus, the oxime of tetrahydroprotomycin gave an acid $C_{10}H_{19-21}COOH$ by Beckmann's rearrangement followed by hydrolysis.

The exactly same procedure applied on streptimidone gave p-bromoazophenacyl ester: orange needles; M.P. 78-81°C; and, empirical formula, $C_{22}H_{26}N_2O_3$. The original acid satisfied the empirical formula of $C_7H_{15}COOH$, as reasonably deduced from the structure of streptimidone.

Treatment of terahydroprotomycin (II) with benzylamine gave a reaction product; white crystals, M.P. 169-17 170°C and molecular formula $C_{21}H_{25}NO_2$. It was identified with the corrosponding product from tetrahydrostreptimidone to establish the presence of -ethyl- -glutarimide moiety.

EXPERIMENTAL

Protomycin (I)-Preparation and properties of protomycin were described in the preceeding papers.

Tetrahydroprotomycin (II)-Protomycin (I) (1.28g) was dissolved in 20 ml of glacial acetic acid, added with 500 mg of 5% Pd-on-Carbon and hydrogenated with stirring at atmospheric pressure and 15°C. Hydrogen (2.03 mol.) was absorbed in 403 min.. The reaction mixture was filtered and evaporated. The residue was dissolved in a small volume of benzene, placed on a column of HCl-treated alumina and developed with ethyl-acetate. The eluate was evaporated and purified with molecular distillation to give a pale yellow viscous liquid. It solidified gradually, when allowed to stand in refrigerator. M.P. 39-44°C.

Anal. Calcd. for $C_{19}H_{33}NO_5$: C, 64.20; H, 9.36; N, 3.94.

Found : C, 64.05; H, 8.98; N, 4.25.

λ_{Max}^{MeOH} =282 mμ (ε =203).

Alkaline degradation of protomycin- Protomycin (461 mg) was dissolved in 4% aq. NaOH, distilled immediately into 2N-HCl, saturated with 2,4-dinitrophenyl-hydrazine. The solution was extracted with benzene and evaporated. The residue was dissolved in a small volume of benzene, placed on a column of silicic acid and developed with the same solvent. The 1st dark red effluent was evaporated and recrystallized from ethylacetate to give a dark red needles. M.P. 165°C.

Anal. Calcd. for $C_{15}H_{18}N_4O_4$: C, 56.69; H, 5.70; N, 17.74.

Found : C, 57.05; H, 5.80; N, 17.74.

It was identified with the corresponding derivative obtained from streptimidone by the same procedure, in referring to mixed melting point and infrared spectrum.

The second band, eluted with 1% ethylacetate in benzene, gave orange crystals, M.P. 122°C.

Anal. Calcd. for $C_9H_{10}N_4O_4$: C, 45.38; H, 4.23; N, 23.52.

Found : C, 45.51; H, 4.27; N, 23.80.

Mixed melting point and infrared spectrum supported it to be the derivative of acetone.

Alkaline degradation of tetrahydroprotomycin - Tetrahydroprotomycin (II) (540 mg) was treated with alkali and steam distilled as described above. The extract of

2,4-dinitrophenyl hydrazone was fractionated through a column of silicic acid. Two bands, moving closely yet in clear separation, were eluted successively. The 1st eluate was evaporated, recrystallized from 95% ethanol repeatedly to give yellow needles, M.P. 59-62°C.

Anal. Calcd. for $C_{15}H_{22}N_4O_4$: C, 55.88; H, 6.88; N, 17.38.
Found : C, 56.32; H, 7.04; N, 17.49.

λ_{Max}^{EtOH} =263 mμ (a=51).

By treating tetrahydroprotomycin in the same way, a similer derivative, M.P. 67-71°C was obtained. The identity between two products was established on the basis of mixed melting point (61-67°C) and infrared spectrum.

The second band gave yellow crystals M.P. 96-98°C.

Anal. Calcd. for $C_{15}H_{22}N_4O_4$: C, 55.88; H, 6.88; N, 17.38.
Found : C, 55.91; H, 6.63; H, 17.36.

Although the band was not obtained from streptimidone, the mixed melting point with the above product was in the intermediate range (77-87°C). The infrared spectrum was identical each other.

The slowly moving red band was eluted with 1% ethylacetate-benzene. Recrystalization from 95% EtOH gave orange needles, M.P. 116-120°C.

Anal. Calcd. for $C_6H_{10}N_4O_4$: N, 23.52.
Found : N, 23.53.

It was identified with the derivative from acetone.

Ozonolysis of protomycin-Protomycin (1.01g) was dissolved in 20 ml methanol and cooled in a bath of ice-sodium chloride. After bubbling through ozone, 20 ml water containing 1g $FeSO_4$ was added to decompose the ozonide, filtered and distilled into 2N-HCl-2,4-dinitrophenylhydrazine, which was extracted with benezen and purified by silicic acid chromatography. Yellow needles with M.P. 158-161°C was obtained.

Anal. Calcd. for $C_7H_6N_4O_4$: N, 26.92.
Found : N, 26.85.

It was identified with an authentic derivative of formaldehyd.

To the residual solution (10 ml) was added 10% NaOH (10 ml) and steam distilled into 2N-HCl-2,4-dinitrophenylhydrazine. The acid solution was extracted with benzene and developed through a column of silicic acid: the first band gave orange crystals, M.P. 106-108°C;

the second band, orange crystals, M.P. 118°C.

Anal. of the former, Calcd. for $C_{10}H_{12}N_4O_4$:
C, 47.62; H, 4.80; N, 22.22.
Found : C, 47.69; H, 4.87; N, 22.01.

It was identified with the derivative of methylethyl ketone by mixed melting point and infrared spectrum. The second was proved to be acetone.

Protomycin (I) (520 mg) in 20 ml chloroform was treated with ozone in an ice-NaCl bath. The solvent was evaporated <u>in vacuo</u> and the residual viscous liquid was decomposed with water, followed by steam distillation. On addition to formaldehyde, acetoaldehyde was found as 2,4-dinitrophenylhydrazone, M.P. 158-160°C.

Anal. Calcd. for $C_8H_8N_4O_4$: N, 24.99.
Found : N, 24.33.

Residual solution was alkalinized and steam distilled to prove again methyl ethyl ketone and acetone.

<u>A C_{11}-acid from tetrahydroprotomycin oxime</u> - A mixture of tetrahydroprotomycin (1.28 g, 3.66 mol), pyridine (290 ml) and hydroxylamine hydrochloride (254 mg) in 10 ml ethanol was refluxed for two hours, evaporated, washed with water and ether, and dried.

Since the residue remained viscous in spite of several treatments, 10 ml. of 80% sulfuric acid was added, heated on boiling water bath for 1 hour, poured into 200 ml. of cold water and steam distilled. The distillate containing floating oily material consumed 2.04 mol equivalent of sodium hydroxide when neutralised with standarised 0.1N NaOH.

The neutralized solution was evaporated <u>in vacuo</u> and refluxed with 500 mg of p-phenylazophenacyl bromide in 90% aqueous ethanol for two hours. The reaction mixture was evaporated to dryness and separated from original reagent by silicic acid chromatography developed with benzene. Twice recrystalization from 95% EtOH gave orange needles, M.P. 54-55°C (VII).

Anal. Calcd. for $C_{25}H_{30}N_2O_3$: C, 73.71; H, 7.79; N, 6.60.
Found : C, 73.86; H, 7.44; N, 6.99.

The elemental analysis suggested the original acid to have an empirical formula of $C_{10}H_{19-20}COOH$.

<u>A C_8-acid from tetrahydrostreptimidone oxime</u>.- The procedure exactly same as in the just preceeding pragraph was repeated, but the protomycin was substituted with streptimidone. The corresponding p-phenylazophenacyl ester was obtained; orange needles, M.P. 78-81°C.

Anal. Calcd. for $C_{22}H_{26}N_2O_3$: C, 72.10; H, 7.15; N, 7.65.
Found : C, 72.13; H, 7.13; N, 7.75.

A reaction product of benzylamine and tetrahydroprotomycin - A mixture of tetrahydroprotomycin (II) (1g) and benzylamine (1.5 ml) was heated on steam bath for four hours, added with carbon tetrachloride to precipitate a solid. The solid was recrystallized from chloroform-ether and hot methanol to obtain a white solid, M.P. 169-170°C.

Anal. Calcd. for $C_{21}H_{25}N_3O_2$: C, 71.20; H, 7.07; N, 11.96.

Found : C, 71.77; H, 7.07; N, 11.46.

Mixed melting point and infrared spectrum established the identity between the corresponding product from streptimidone and the just obtained one.

ACKNOWLEDGEMENT

The author wishes to express sincere thanks to Dr. Toju Hata, Chief of Antibiotic Division of Kitasato Institute and Dr. Masanao Matsui for their instructions and encouragement; to Dr. John Ehrlich, Parke Davis and Company for donating streptimidone; to the members of Fuji Branch, Kyowa Hakko Kogyo Company for supplying the materials; and to the member of laboratory of elemental analysis in Sankyo Company. The research was supported by the Fund from U.S. Army Research and Development Group (9852) (Far East).

LITERATURES

(1) Sugawara, R., A. Matsumae and T. Hata: Protomycin, new antibiotic of actidione group, II Production and properties. (in press).

(2) Frohardt R. P., H. W. Dion, Z. L. Jakubowski, A. Ryder, J. C. French and Q. R. Bartz: Chemistry of Streptimidone, a new antibiotic. J.A.C.S., 81, 5500 (1959).

(3) Van Tamelen E. E. and V. Hearstad: Structure of the antibiotic streptimidone. J.A.C.S., 82, (2974), 1960.

(4) Woo, P. W. K., H. W. Dion and Q. R. Bartz: The structure of streptimidone. J.A.C.S., 83 (3085), 1961.

b. Cephalomycin, further purification

It has been found that not a few antibiotics isolated from streptomyces and other species of microorganisms, such as helenin(1), myxoviromycin(2), abikoviromycin(3) and others, have antiviral and antiphage activities.

During screening for antiviral antibiotics in our laboratory, the strain, Streptomyces tanashiensis var. cephalomycetious, which produces an active substance against Japanese B. encephalitis virus in vivo, was isolated from a soil sample obtained in Tokyo. This active product was concluded to be a new substance and was named cephalomycin by Hata et al. Matsumae and Onuma reported mycological characteristics of the producing strain, biological and chemical properties of cephalomycin(4), and activities against J. encephalitis virus in mice(5). According to those previously reported data, cephalomycin, although not homogeneous, was supposed to be an acidic polypeptide, being precipitated at the pH range 2-5 as a brownish amorphous powder. It is non-dialysable, insoluble in organic solvents, and is inactivated when incubated with trypsin. It is positive in Sakaguchi, biuret, ninhydrin, diazo and Folin's color reactions. Acid hydrolysates gave ninhydrin positive spots by paper chromatography.

Cephalomycin was not only active against Japanese B. encephalitis virus in the contact experiment, but was effective if given within 24 hours in advance to inoculation of Japanese B. encephalitis virus. Also, infected mice which have survived by treatment with cephalomycin gained a significant immunity against Japanese encephalitis reinfection.

The object of the present work is to isolate a further purified preparation of cephalomycin and to investigate its physicochemical and ultimately its biological properties.

EXPERIMENTAL

Organism: The cephalomycin producing strain, Streptomyces tanashiensis var. cephalomycetious, maintained in our laboratory, was employed for preparing cephalomycin.

Medium: An aqueous medium, containing 2.0% starch, 1% soy bean meal, 0.5% peptone, 0.5% meat extract, 0.4% dry yeast, 0.5% NaCl and 0.3% $CaCO_3$, initial pH 7.0, was used for cephalomycin producing medium.

Animals: dd mice of both sex, weighing about 10 gr were used.

Virus: The strain Nakayama of Japanese B. encephalitis virus, intracerebrally transfered in our laboratory, was used.

Assay method of cephalomycin: Samples were assayed for cephalomycin activity by a technique essentially the same as that described by Matsumae and Onuma; when Japanese B. encephalitis virus was injected to mice intracerebrally with 10^{-7} – 10^{-8} virus concentrations, the mice showed typical symptoms of encephalitis after 3 to 5 days after inoculation. However, in this experiment, intraperitoneal injection was used for convenience. In the case of intraperitoneal inoculation of virus, the typical symptoms appeared in mice with virus concentrations of $10^{-1.5}$ – $10^{-2.5}$. Accordingly, 10^{-1} virus concentration was used for the experiment. The brains of symptomatic mice were removed and collected aseptically and homogenized in a homogenizer at 18,000 r.p.m. with Hank's solution added to the ratio of 4 ml. per 3 brians. This homogenate was centrifuged at 3,000 r.p.m. for 5 min. to remove brain debris and the supernatant solution ($10^{-0.5}$ virus concentration) was mixed with an equal volume of appropriate concentration of cephalomycin solution to give 10^{-1} virus concentration. This mixed solution was allowed to stand in ice water to cause contact virus with cephalomycin. After 15 min. the suspension was injected to mice intraperitoneally. At the same time, the solution of 10^{-1} virus concentration which contained physiological saline instead of cephalomycin was injected to other mice as control experiment. This control group showed typical symptoms of encephalitis. Usually, the symptoms of Japanese encephalitis appeared after about 4 days and the result become definite within 8 days, but the mice were observed still further for 14 days following the inoculation. Therefore, if death occurred within 4 days, it was regarded as an accident or non encephalitic death and in this case the mice were excluded from the experiments. Cephalomycin activity was represented in terms of the ratio of survivied mice to treated ones.

Fermentation: St. cephalomyceticus was inoculated to above described medium and cultured for 96 hrs at 27°C.

Following procedures were applied for further purification of cephalomycin.

(1) Purification of cephalomycin

(a) Isoelectric precipitation: The 96 hour fermentation liquor of St. cephalomyceticus, separated from mycelia by centrifugation, was cooled with ice and adjusted to pH 3.6 by adding gradually 10% aqueous acetic acid with stirring. A heavy precipitate formed, and, after 15 min. of further stirring, the precipitate was collected with a sharples centrifuge. The inactive supernatant was discarded, and the precipitate was dissolved, with cooling, in distilled water by adjusting the pH to 8 with aqueous ammonia. The resulting solution was centrifuged and insoluble precipitate was discarded. The supernatant solution was readjusted to pH 3.6 with 10% acetic acid to precipitate cephalomycin. This precipitate was centrifuged and washed with aqueous acetic acid (pH 3.6) repeatedly. After the washings became clear, the isoelectric precipitate of cephalomycin was dissolved in distilled water by adjusting pH to 7.0. This procedure was repeated twice; finally, a red-purple colored cephalomycin solution was obtained.
(b) Sephadex, a recently developed material for purifying macromolecular substances, was employed. Sepahdex G-25 was suspended in distilled water, lighter floating gel particles separated, and the gelled Sephadex packed into a glass column. To the top of well washed Sephadex column, thecephalomycin solution obtained by isoelectric precipitation was poured on carefully. After the solution had been soaked into Sephadex completely, elution was carried out with distilled water. Cephalomycin solution was separated into primarily eluted brown colored band and the following two red colored bands (Fig. 1). Each eluted band was lyophilized. Fig. II and III shows absorption spectrum of brown and red fractions of cephalomycin. The ultraviolet and visible light absorption spectrum of the brown fraction exhibited a shoulder at the range from 255 to 275 millimicron in 0.1 N-HCl solution and this shifted to the range from 260 to 290 millinicron in 0.1 N-NaOH; probably due to the presence of tyrosine in cephalomycin molecule. The slowly eluted two red fractions had the same absorption spectrum exhibiting a peak at 405 millimicron. Antiviral activity existed in brown fraction (Table II).

(c) Chromatography on DEAE-cellulose: DEAE-cellulose, 2g in day weight, was thoroughly washed with 0.01 M phosphate buffer, pH. 7.0, and packed into a glass column. 40mg of lyophilized powder of the brown fraction was dissolved in 1.5 ml of 0.01 M phosphate buffer, pH 7.0, and was applied to the column. The column was mounted above a fraction-collector and 80 drop (4 ml) fractions were collected. Elution velocity was 16 ml/hr. As shown in Fig. IV, cephalomycin was eluted batchwise with increasing concentrations of phosphate and NaCl. The appearance of cephalomycin in the eluate was estimated by the optical absorption density at 280 millimicrons. After the elution with the phosphate buffers had been finished, 0.2% NaOH solution was passed through the column. Then, still another fraction was eluted. Each eluate was desalted by passing through Sephadex G-25 column, and lyophilized. As a result, cephalomycin was separated to four fractions by DEAE-cellulose chromatography. The antiviral activity was associated with every fractions. The finally eluted fraction had a higher level of activity than others; The first eluate was less brown colored than others.

In order to purity cephalomycin further, several attempts were made, including gradient elution from DEAE-cellulose with a variety of NaCl gradient, rechromatography on DEAE-cellulose, chromatography on TEAE- and ECTEOLA-cellulose, calcium phosphate gel chromatography and starch gel electrophoresis. All these methods failed in increasing unitary activity of cephalomycin. The above described purification procedure was summerized in Table I.

(2) Some physicochemical and biological properties of cephalomycin

(a) Paper electrophoresis: Paper electrophoresis was carried out with horisontal method, using Toyo Roshi No. 51 filter paper, 2 x 30 cm., M/20 veronal buffer, pH 8.6, in the conditions of 100 v., 5 hrs., 0.1 mA/cm. The color was developed with 1% bromphenol blue solution followed by washing with 2% acetic acid.

As shown in Fig. V, the original iscelectric precipitate was separated into 3 fraction as reported previously. The red fraction after cephadex eluted from Sephadex contained only a small amount of protein

and remained nearly at the original point; The brown fraction was separated into 3 fractions. The fractions eluted from DEAE-cellulose chromatography moved to different positions respectively.

(b) Antiviral activity: Table II represents antiviral activities of the purified powders of cephalomycin, determined by the above described methods. There was no activity in the red fraction, but the brown fraction revealed a higher activity than the original isoelectric precipite. As for four fractions separated by the chromatography on DEAE-cellulose, no remarkanle difference of antiviral activity was proved between one another, but when compared with the potency of isoelectric precipite, the activity increased at least twofold.

(c) Amino acid analysis: One fraction of cephalomycin, eluted by 0.2% NaOH from DEAE-cellulose column chromatography, was hydrolysed in 6 N-HCl for 24 hrs., at 115°C, dryed up in vacuo repeatedly, with added water in order to remove residual HCl. The residue was dissolved in water and analysed by two-dimensional paper chromatography with solvent system of butanol-acetic acid-water (4:1:2) and phenol-water (4:1), and by modified finger print method of Ingram. The modified finger print technique was as follows: a sample of hydrolysate was spoted in a middle side of 40 x 40 cm. filter paper, and electrophoresis was carried out toward both sides of the spotted point, followed by paper-chromatographic development (butanol-acetic acid-water, 4:1:2) to another direction. Amino acids spots were colored by spraying ninhydrin solution.

A part of hydrolysate was mixed with 2% dinitrofluorobenzen solution and 50% ethanol containing 4% $NaHCO_3$ to dinitrophenylate. The resultant dinitrophenylated amino acids (DNP-amino acids) were extracted with ethyl ether and butanol, and were analysed by two-dimensional paper chromatography (5% aqueous ammonia saturated butanol and 1.5 M phosphate).

As indicated in Fig. VI and VII, the following amino acids were identified: aspartic acid, glutamic acid, arginine, lysine, histidine, cystine, glycine, serine, threonine, alanine, proline, tyrosine, valine, leucine, phenylalanine and methionine.

The spots of DNP-amino acids on paper were out out, extracted with 5 ml. of 1% $NaHCO_3$ solution,

and extracts were estimated by optical density at 360 millimicron. The molar ratio of each DNP-amino acids were: glycine 2.8, serine 2.7, glutamic acid + aspartic acid 7.8, threonine 2.0, proline 1.6, alanine + methionine 3.5, leucine + valine 8.9, phenylalanine 1.6, histidine 1.1, cystine 0.2 and tyrosine + lysine 2.6.

Cephalomycin, eluted from DEAE-cellulose chromatography with 0.2% NaOH, was dinitrophenylated, and DNP-cephalomycin was hydrolysed in 6 N-HCl for 14 hrs. at 110°C. As showed in Fig. VIII, presumable N-terminal amino acid of this cephalomycin was an acidic amino acid (aspartic acid or glutamic acid).

DISCUSSION

According to the above described data, cephalomycin was characterized as a protein-like substance and several conventional methods of protein purification were applied for purification of cephalomycin. The antiviral activity was, however, not so markedly increased, but was separated into several fractions with almost similar activity levels. It has been propsed that intact and strictly regulated structure of large protein molecule was essential for enzymes or active proteins to exhibit a complete activity. On the other hand, it has also been proposed that enzymes or active proteins has a one or more active center parts in molecules, which consists of some amino acid residues with limited steric structure, to exhibit activity, as have been demonstrated for several enzymes and active proteins such as trypsin, pepsin, insulin and chyrochrome c. In this connection, cephalomycin may be considered also to have active parts in its molecules: the DEAE-cellulose chromatography separated cephalomycin into several fractions with almost same degrees of activity, thereby suggesting a possibility that each fractionated cephalomycin have a common antiviral active part in their molecules, and the other parts of molecules have different arrangements of amino acids; or, St. cephalomyceticus produces only one active substance in culture broth, and simultaneously produces proteolytic enzymes for them and the latter attacks some parts of the former, allowing an active part unaffected to give several molecules with a common antiviral active center. In the fractionation experiments with chromatography on DEAE-cellulose and paperelectrophoresis, it was often found that the number and location of the active fractions somewhat varied from lot to lot. This variation would suggest the above described possibility.

SUMMARY

1. Cephalomycin was isolated from the culture broth by isoelectric precipitation, gel filtration on Sephadex and chromatography on DEAE-cellulose.

2. Purified preparation was shown to be homogeneous by paperelectrophoresis.

3. Each separated fraction of cephalomycin exhibited the almost same degree of antiviral activity.

4. Amino acid analysis and presumable N-terminal amino acid of one fraction of cephalomycin were determined.

ACKNOWLEDGEMENT

The authors wish to thank Prof. K. Satake and Dr. S. Sasakawa of Department of Chemistry, Tokyo Metropolitan University, for their kind guidance.

This work was supported by the grant from U.S. Department of Army, Far East Research Office.

LITERATURES

(1) R. E. Shope: An antiviral substance from Penicillium funiculosum, I and II, J. Exp. Med., 97, 601, 627 (1953).

(2) M. Kuroya, et al.: Studies on antiviral antibiotics from streptomyces. A new antiviral antibiotic, myxoviromycin. Jap. J. Microbiol., 1, 85 (1957)

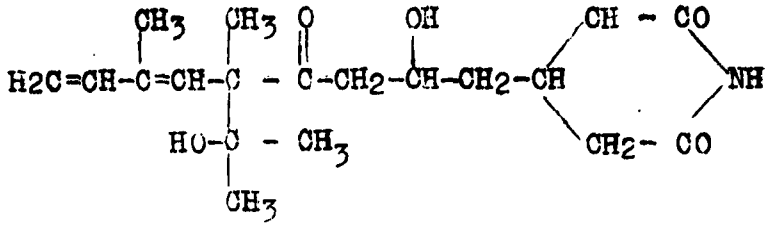
(3) H. Umezawa, et al.: An antiviral substance, abikoviromycin, produced by streptomyces species, Jap. Med. J., 4, 331 (1951).

(4) A. Matsumae.: Cephalomycin, a new antiviral antibiotic, I. J. Antibiot., Ser. A, 13, 143 (1960).

(5) M. Onuma; Cephalomycin, a new antiviral antibiotic. II. J. Antibiot., Ser. A., 13, 273 (1960)

SUMMARY

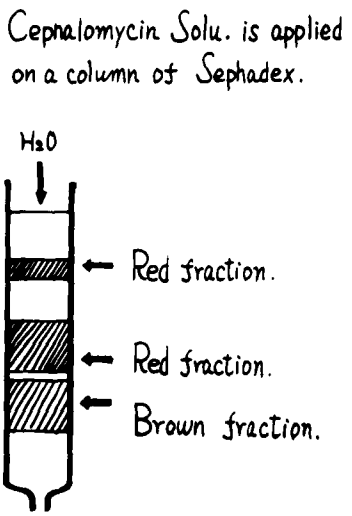
Protomycin is a new antibiotic belonging to cycloheximide group with activities against Endameba histolytica and saccharomyces, particularly resembling to streptimidone by Frohardt et al in Parke Davis Company. Degradation products of protomycin were compared with corresponding ones simultaneously obtained from streptimidone. The following structure of protomycin was proposed on the basis of the products in referring to the established structure of streptimidone:



Purification of cephalomycin was carried out with isoelectric precipitation and chromatography on Sephadex and DEAE cellulose. The unitary activity increased twice. The amino acid constitution (glycine, serine, glutamic and aspartic acids, threonine, proline, alanine, methionine, leucine, valine, phenylanine, histidine, cystine, tyrosine and lysine) and N-terminal amino acid (aspartic or glutamic acid) were determined.

APPENDIX 1

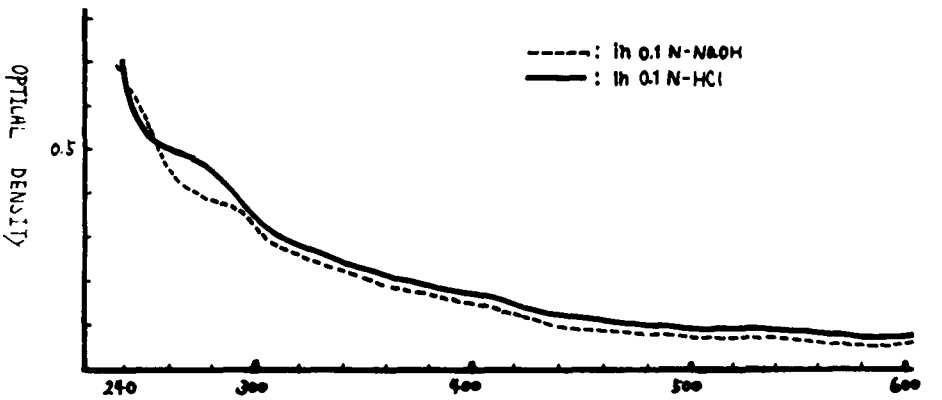
Fig. 1.
Separation on a Column of Sephadex G-25



APPENDIX 2

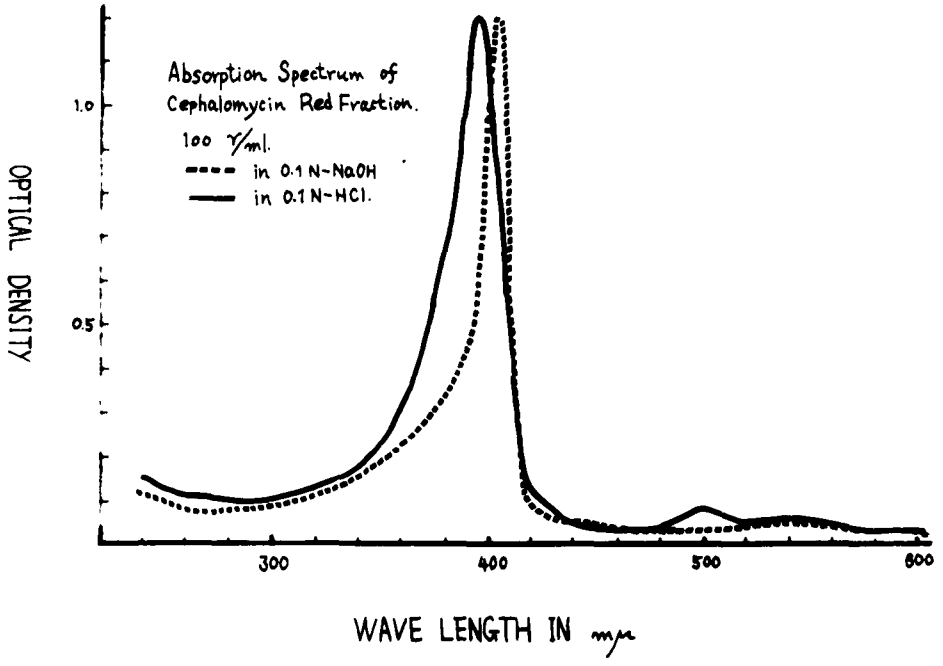
Fig. II.

Absorption Spectrum of Cephalomycin Brown Fraction



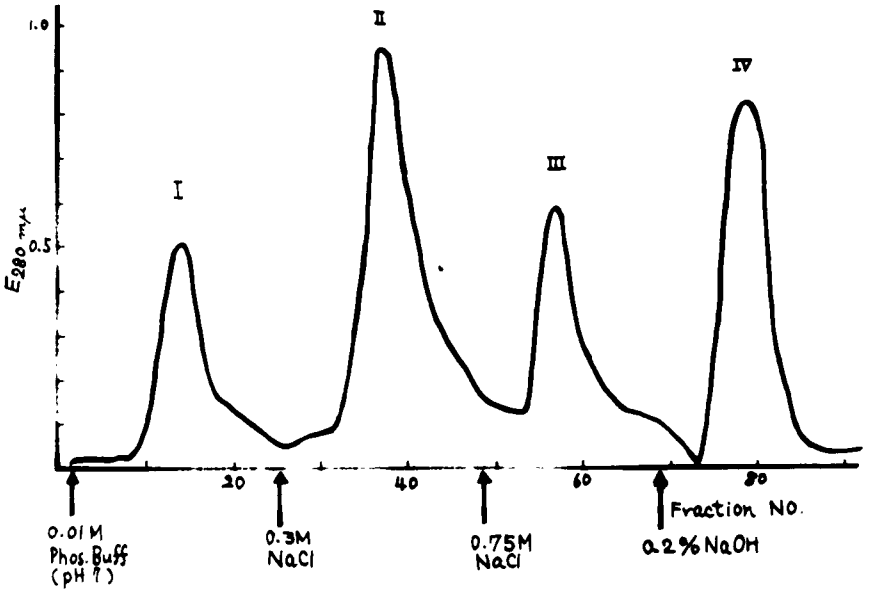
APPENDIX 3

Fig. III



APPENDIX 4

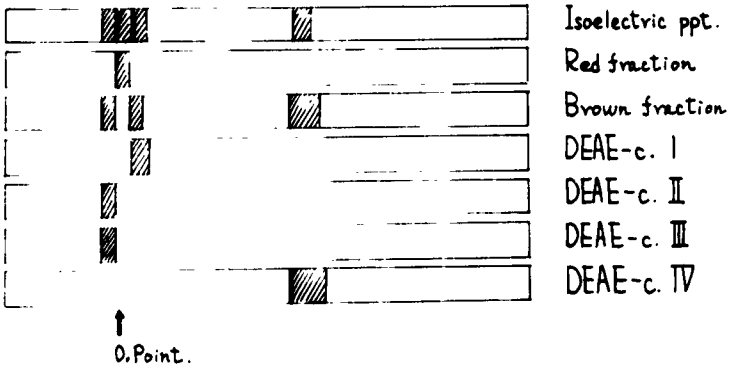
Fig. IV
Elution of Cephalomycin from DEAE-cellulose



APPENDIX 5

Fig. V Paper Electrophoresis of Cephalomycin

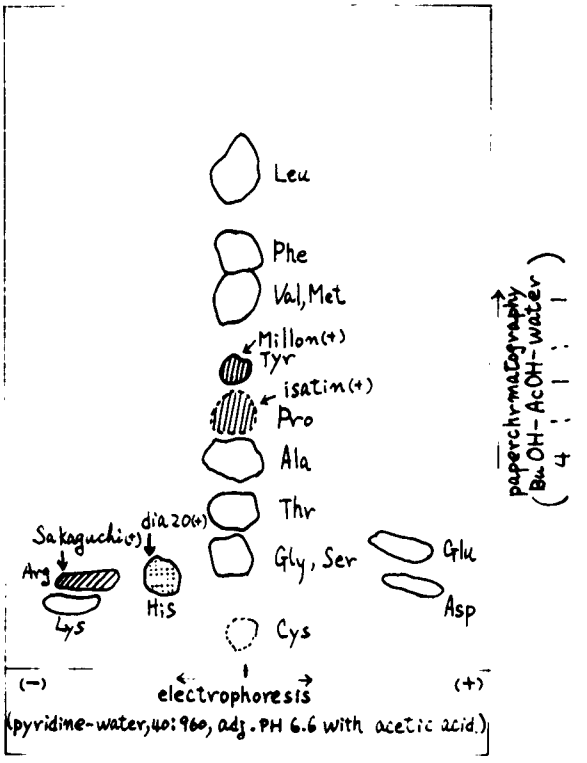
100 v. 5 hrs. 0.1 mA/cm
Paper : Toyo Roshi No.51 2x30 cm
Buffer: Veronal pH 8.6 (μ=0.05)
Color : B.P.B 1% solu



APPENDIX 6

Fig. VI

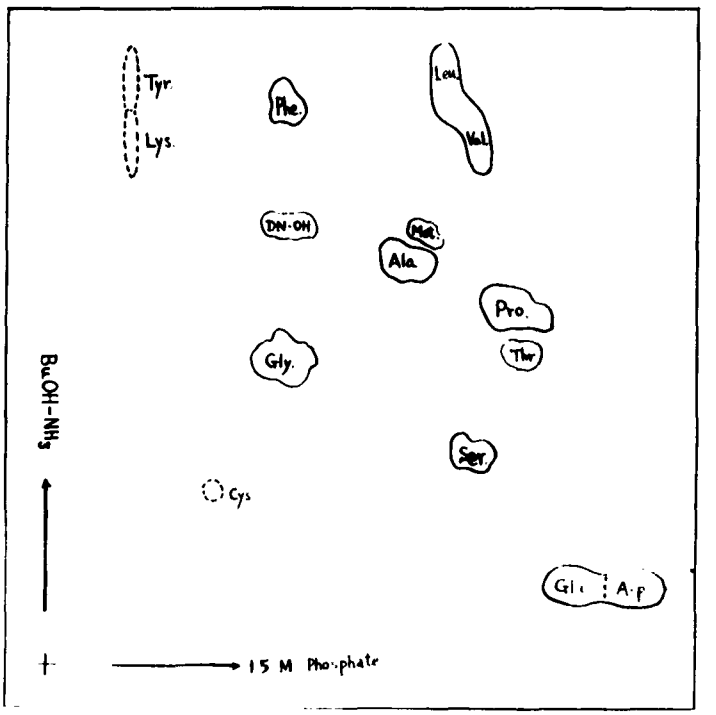
Paperelectro-chromatogram of amino acid in cephalomycin (DEAE-c, 0.2% eluate)



APPENDIX 7

Fig. VII

DNP-Amino acids chromatogram of cephalomycin (DEAE-c., NaOH eluate) hydrolysate

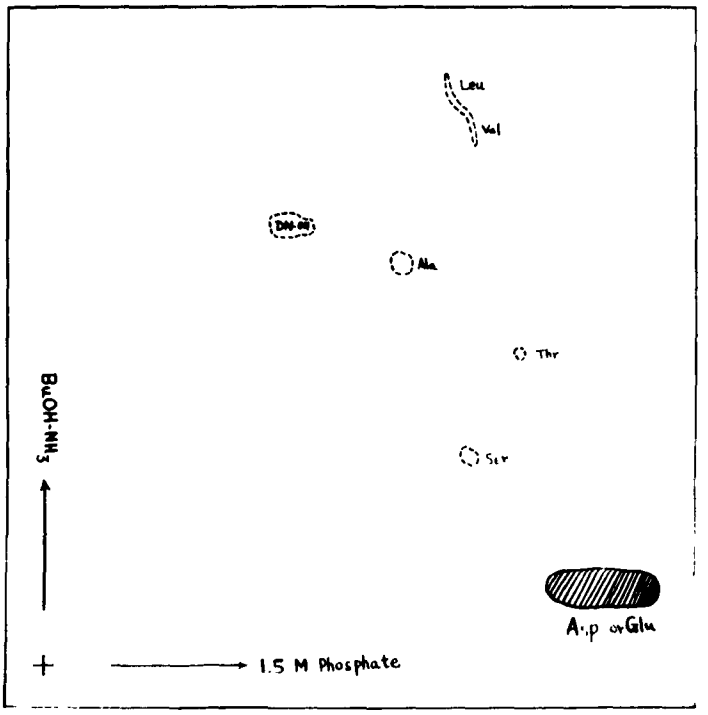


APPENDIX 8

Fig. VIII

N-terminal amino acid of

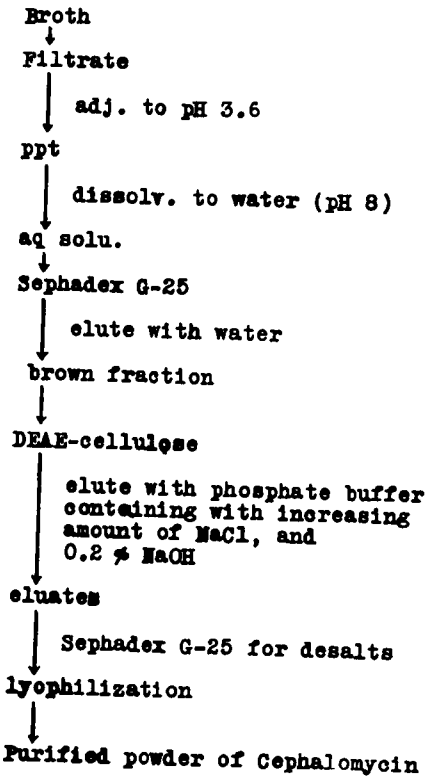
Cephalomycin (DEAE-c. 0.2 %NaOH eluate)



APPENDIX 9

Table I

Purification procedure of Cephalomycin



APPENDIX 10

Table II
Antiviral activity of purified Cephalomycin

Cephalomycin conc. (mcg/ml)	isoelectric ppt.	red fraction	brown fraction	DEAE-cellulose (I)	DEAE-cellulose (II)	DEAE-cellulose (III)	DEAE-cellulose (IV)
	Animal surviving / Animal tested.						
500	5/5	2/5	5/5	5/5	5/5	5/5	5/5
250	4/5	1/5	5/5				
125	4/5	1/5	4/5	4/5	5/5	3/5	4/5
62	3/5	0/5	5/5				
31	2/5	1/5	4/5	3/5	3/5	4/5	4/5
8	2/5	0/5	3/5				4/5
4	0/5		1/5				
control.	0/5	0/5	0/5	0/5	0/5	0/5	0/5